Preliminary Study Protocol

The 3R Senolytic Approach

Fisetin + NMN + Spermidine in Pulsed Administration

Targeting Cellular Senescence in Healthy Volunteers

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1. Background — What is Cellular Senescence?

1.1 The "Zombie Cells" in Your Body

Imagine a cell in your body that has been damaged by sunlight, pollution, or simply by living for a long time. In a healthy scenario, this damaged cell would do one of two things: repair itself, or quietly die (a process called *apoptosis*) to make room for a fresh new cell.

But sometimes, a third thing happens. The cell is too damaged to repair itself, but it **refuses to die**. It just sits there — alive but unable to divide. We call these cells **senescent cells**, but scientists sometimes call them "zombie cells" because they're technically alive but functionally useless.

> 💡 **Simple Explanation**
>
> Senescent cells are like workers who are too tired to work but won't retire or leave the office. They just sit at their desks, doing no useful work, and worse — they complain constantly, making everyone around them miserable too.

1.2 How Does a Cell Become Senescent?

Every time a cell divides, the little caps at the end of its DNA (called **telomeres**) get a tiny bit shorter. Think of telomeres like the plastic tips on shoelaces — they protect the DNA from fraying. After about 50 divisions (the "Hayflick Limit"), the telomeres become too short, and the cell stops dividing. This is one way to become senescent.

But there's a faster way: **DNA damage**. When UV rays from the sun, reactive oxygen species (ROS), or other stressors damage a cell's DNA, the cell activates a defense mechanism. Special proteins called **p53** and **p21** hit the brakes on cell division to prevent the damaged DNA from being copied. If the damage is too severe to fix, another protein called **$p16^{INK4A}$** locks the cell into a permanent state of arrest. The cell becomes senescent.

```
DNA damage → p53 activated → p21 (temporary stop) → if damage persists → p16^INK4A (permanent stop) = SENESCENCE
```

1.3 SASP — The Toxic Secretion

Here's the worst part. Senescent cells don't just sit there silently. They secrete a toxic cocktail of molecules called the **SASP (Senescence-Associated Secretory Phenotype)**. The SASP contains:

- **Pro-inflammatory cytokines** (IL-6, IL-8): these cause chronic, low-grade inflammation ("inflammaging")
- **Proteases** (MMP-1, MMP-3): these chew up collagen and destroy the skin's structure
- **Growth factors** that can disrupt normal tissue function

The SASP is contagious — it pushes neighboring *healthy* cells to also become senescent. This creates a snowball effect where more and more cells become zombies over time.

> 💡 **Simple Explanation**
>
> One zombie cell secretes chemicals that turn its healthy neighbors into zombies too. Over years and decades, the zombie population grows, slowly damaging entire tissues and organs.

1.4 Why This Causes Aging and Disease

As senescent cells accumulate with age, they contribute to almost every age-related disease:

- **Skin**: senescent fibroblasts cause brown spots, wrinkles, and loss of elasticity
- **Arteries**: senescent endothelial cells contribute to atherosclerosis
- **Brain**: senescent microglia and astrocytes drive neuroinflammation (Alzheimer's, Parkinson's)
- **Joints**: senescent chondrocytes cause osteoarthritis
- **Pancreas**: senescent beta-cells contribute to Type 2 diabetes

1.5 The Yoon Discovery (2018)

In 2018, Yoon et al. made a landmark discovery. They took skin biopsies from brown spots (solar lentigines) on the faces of patients aged 55-62 and compared them to normal skin from the same patients. They found that:

- Brown spots were enriched with **senescent fibroblasts**
- These fibroblasts had low levels of **SDF1** (Stromal Cell-Derived Factor 1), a protein that normally tells melanocytes to stop producing pigment
- The senescent fibroblasts themselves had abnormally high **tyrosinase** activity (the enzyme that makes melanin)

Critically, when they eliminated the senescent fibroblasts using microneedle radiofrequency, **the brown spots faded significantly**. This was the first experimental proof that senescent cells are the *cause*, not just a consequence, of age-related pigmentation.

2. Why This Study? — The 3R Hypothesis

2.1 The Three Rs

Based on the science of cellular senescence, we propose a three-pronged approach called **3R**:

REMOVE — Kill the zombie cells

Fisetin (a natural polyphenol from strawberries) is the most potent natural senolytic identified. It works by inhibiting the anti-death proteins (SCAP network) that keep senescent cells alive. Without these proteins, senescent cells finally undergo apoptosis. Healthy cells are unaffected because they don't rely on SCAP for survival.

REPAIR — Prevent new zombies from forming

NMN (Nicotinamide Mononucleotide) is a precursor to NAD+, a critical molecule that drops by 50% as we age. NAD+ is needed for DNA repair enzymes (PARPs) and longevity proteins (sirtuins). By restoring NAD+ levels, we help cells repair DNA damage before they're forced into senescence.

REGENERATE — Clean up the mess

Spermidine (from wheat germ, shiitake mushrooms) is the most powerful natural inducer of *autophagy* — the cell's recycling system. Once zombie cells are killed, their debris must be cleaned up. Spermidine activates this cleanup independently of other pathways.

> **Simple Explanation**
>
> Think of it like a city with abandoned, dangerous buildings (senescent cells):
>
> **REMOVE** = Demolish the abandoned buildings (fisetin)
> **REPAIR** = Fix the buildings that are still standing but damaged (NMN)
> **REGENERATE** = Clear the rubble and recycle the materials (spermidine)

2.2 What Are We Testing?

This study asks a simple question: **Does the 3R combination, given in intermittent pulses, reduce the burden of senescent cells and improve health markers in humans?**

This has never been tested. Fisetin has been studied in mice (where it extended lifespan and improved health), and early human trials at the Mayo Clinic are encouraging. NMN has been tested separately in small human studies. Spermidine has strong epidemiological data. But the **combination of all three in a pulsed protocol has never been attempted**.

3. Study Design Explained Simply

3.1 Type of Study

This is a **randomized, double-blind, placebo-controlled trial**. These are the gold standard words in clinical research. Here's what each means:

> **Simple Explanation**
>
> **Randomized**: Participants are assigned to the treatment group or the placebo group by a coin flip (actually by a computer). This ensures that the two groups are similar in age, health, etc. If we let people choose, the results would be biased.
>
> **Double-blind**: Neither the participants nor the researchers know who is getting the real treatment and who is getting the placebo. This prevents wishful thinking from influencing the results. Only at the very end do we reveal who was in which group.
>
> **Placebo-controlled**: One group receives identical-looking capsules with no active ingredients. This is the "control" group. If we see improvements only in the treatment group, we know it's because of the supplement, not because of the psychological effect of taking a pill.

3.2 Participants

Criteria	Details
Total number	40 volunteers
Age range	50-75 years old
Treatment group	20 people
Placebo group	20 people
Health status	Generally healthy, no major chronic diseases
Sex	Both men and women (aim for balance)

3.3 Duration

The treatment period lasts **16 weeks (4 months)**. This allows for approximately 7 treatment pulses (2 days on, 13 days off, repeated). We chose 16 weeks because:

- It's long enough to see changes in biomarkers
- It's short enough to keep participants motivated
- Skin cell turnover takes ~28 days, so 4 months = ~4 full renewal cycles

3.4 The Pulse Concept

We don't give the supplements every day. Instead, we use **pulses**: 2 days of high-dose treatment followed by 13 days of maintenance dose. Here's why:

> 💡 **Simple Explanation**
>
> Fisetin is like a sniper — it needs to hit hard and fast to kill zombie cells, then stop. If given continuously, it might accidentally harm healthy stem cells. The 2-day pulse is enough to trigger apoptosis of senescent cells. The 13-day break allows the body to recover and clean up.
>
> Meanwhile, NMN and spermidine are given continuously because their jobs (DNA repair and cleanup) are ongoing.

4. The Treatment Protocol

4.1 Group Assignment

Participants are randomly assigned to one of two groups. Neither the participant nor the researcher knows which group they're in.

4.2 Treatment Group Schedule

Pulse Days (2 consecutive days, every 15 days)

Supplement	Dose	Purpose
Fisetin (GMP grade)	500 mg	Senolytic — kills zombie cells (REMOVE)
Piperine (black pepper extract)	10 mg	Boosts fisetin absorption 5-10×
NMN (Nicotinamide Mononucleotide)	250 mg	NAD+ precursor (REPAIR)
Spermidine	3 mg	Autophagy inducer (REGENERATE)

Maintenance Days (the other 13 days)

Supplement	Dose
NMN	250 mg daily
Spermidine	3 mg daily
Fisetin	— (not given)

4.3 Placebo Group Schedule

The placebo group receives identical-looking capsules containing **microcrystalline cellulose** (a harmless filler used in virtually all supplement and pharmaceutical capsules). The capsules look, smell, and taste the same. The packaging is identical. The schedule (pulse days vs. maintenance days) is the same.

4.4 Visual Pulse Calendar (16 weeks)

Each row = 2-week cycle. **[FF]** = Fisetin + full dose pulse (days 1-2). **[..]** = maintenance (days 3-14).

```
Week 1-2: [FF] [.....] Week 3-4: [FF] [.....] Week 5-6: [FF]
[.....] Week 7-8: [FF] [.....] Week 9-10: [FF] [.....]
Week 11-12: [FF] [.....] Week 13-14: [FF] [.....] Week 15-16:
[FF] [.....] Total: 7 pulses × 2 days = 14 days of fisetin over 16 weeks
```

4.5 Administration Instructions

- Take all capsules with food (improves absorption, reduces stomach upset)
- Take in the morning (NMN may cause alertness similar to caffeine)
- On pulse days: take the pulse capsules AND the maintenance capsules
- A calendar with clearly marked "PULSE DAYS" is provided to each participant

5. What We Measure (Biomarkers)

A biomarker is a measurable signal in the body that tells us what's happening at the biological level. We measure several biomarkers at 5 time points during the study.

5.1 Measurement Schedule

Visit	Timing	What Happens
Visit 1	Day 0 (baseline)	Blood draw + clinical measurements BEFORE any treatment
Visit 2	Day 30	Blood draw (after ~2 pulses)
Visit 3	Day 60	Blood draw (midpoint, after ~4 pulses)
Visit 4	Day 90	Blood draw (after ~6 pulses)
Visit 5	Day 120	Final blood draw + clinical measurements

5.2 Primary Biomarkers

$p16^{INK4A}$ and p21 (mRNA levels in blood)

These are the "ID badges" of zombie cells. When cells become senescent, they produce high levels of p16 and p21. We measure the mRNA (the genetic message that produces these proteins) in a small blood sample using a technique called **qPCR** (quantitative Polymerase Chain Reaction).

> **Simple Explanation**
>
> **qPCR explained**: DNA contains instructions. mRNA is the "working copy" of those instructions. If a cell has lots of p16 mRNA, it means it's actively producing p16 protein — which means it's senescent. qPCR is a machine that counts how many copies of a specific mRNA are in your sample.

Expected result: p16 and p21 levels should **decrease** in the treatment group vs. placebo.

SA-β-galactosidase (optional skin biopsy)

This is the classic "gold standard" marker for senescence. It's an enzyme that senescent cells produce in large amounts. We can detect it by adding a special chemical (X-Gal) that turns **blue** when cut by the enzyme. The more blue cells, the more senescent cells.

This test is performed on a tiny 3mm skin punch biopsy (optional, only for participants who consent).

SASP Markers: IL-6 and IL-8 (blood, by ELISA)

IL-6 and IL-8 are inflammatory cytokines — key components of the toxic SASP cocktail secreted by zombie cells. We measure them using **ELISA** (Enzyme-Linked Immunosorbent Assay).

> **Simple Explanation**
>
> **ELISA explained**: We put a drop of blood serum into a special plate. The plate contains antibodies that specifically grab onto IL-6 (or IL-8) like a lock and key. Then we add an enzyme that changes color when IL-6 is present. The more intense the color, the more IL-6 there is. A machine called a microplate reader measures the color intensity precisely.

Expected result: IL-6 and IL-8 should **decrease** in the treatment group.

NAD+ Levels (blood)

NAD+ is the molecule that NMN is supposed to boost. We need to verify that our NMN supplementation actually increases NAD+ levels in the blood. This is measured by **HPLC** (High-Performance Liquid Chromatography) or a specialized NAD+ assay kit.

> **Simple Explanation**
>
> **HPLC explained**: Imagine a very sophisticated filter that separates molecules by size and chemical properties. NAD+ is one specific molecule among thousands in blood. HPLC separates it from everything else and counts exactly how much is there.

Expected result: NAD+ should **increase** in the treatment group.

Autophagy Markers: LC3-II/I ratio and p62 (blood)

Autophagy is the cell's recycling process. **LC3-II** is a protein that forms on autophagy membranes — high LC3-II means active autophagy. **p62** is degraded during autophagy — low p62 means active autophagy.

These are measured by **Western blot** or ELISA on blood cells.

> **Simple Explanation**

> **Western Blot explained**: Proteins are separated by size using electricity (they move through a gel — small proteins move fast, large proteins move slowly). Then we use antibodies to "light up" only the protein we care about (like LC3 or p62). The brightness tells us how much is there.

Expected result: LC3-II/I ratio should **increase**; p62 should **decrease**.

SDF1 / CXCL12 (blood, by ELISA)

SDF1 is the protein discovered by Yoon et al. (2018) to be low in senescent skin tissue. It normally tells melanocytes to stop producing pigment. We measure it to see if the 3R approach restores SDF1 levels.

Expected result: SDF1 should **increase** in the treatment group.

5.3 Summary of Expected Changes

Biomarker	Direction	Why
$p16^{INK4A}$	↓ Decrease	Fewer senescent cells
p21	↓ Decrease	Fewer senescent cells
IL-6	↓ Decrease	Less SASP (less zombie toxin)
IL-8	↓ Decrease	Less SASP
NAD+	↑ Increase	NMN supplementation working
LC3-II/I ratio	↑ Increase	Spermidine activating autophagy
p62	↓ Decrease	Active autophagy degrading it
SDF1	↑ Increase	Sirtuins restored → SDF1 production resumes

6. Clinical Endpoints (Real-World Outcomes)

Biomarkers tell us what's happening inside the body. But we also want to know: **can participants actually feel or see a difference?** Clinical endpoints are measurements that directly reflect how a person looks, moves, and feels.

6.1 Skin Assessment

Measurement	How	What We're Looking For
Brown spot area and intensity	Standardized facial photography (same camera, same lighting, same angle every time)	Reduction in spot size and darkness
Skin elasticity	Cutometer (a small device that gently sucks the skin and measures how it bounces back)	Improved elasticity
Skin hydration	Corneometer (measures skin moisture)	Increased hydration

6.2 Mobility and Physical Function

Test	How	What It Measures
6-Minute Walk Test	Participant walks as far as possible in 6 minutes	Cardiovascular fitness and leg strength
Grip Strength	Hand dynamometer (squeeze a device)	Muscle strength (grip strength correlates with overall health)
Gait Speed	Time to walk 4 meters at normal pace	Mobility (slow gait predicts frailty)
Chair Stand Test	Stand up and sit down 5 times as fast as possible	Leg strength and balance

6.3 Cognitive Assessment

MoCA (Montreal Cognitive Assessment): A 30-point test that takes ~10 minutes. It evaluates memory, attention, language, and orientation. It's the standard screening tool for mild cognitive impairment.

Expected result: Stable or slightly improved scores in the treatment group.

6.4 Quality of Life

SF-36 (Short Form Health Survey): A validated 36-item questionnaire that measures 8 dimensions of health: physical function, bodily pain, general health, vitality, social functioning, emotional role, mental health, and physical role.

Participants complete this at baseline (Day 0) and Day 120.

6.5 Adverse Event Monitoring

Every participant receives a **daily symptom diary** (paper or app) to record:

- Any unusual symptoms (nausea, headache, fatigue, digestive issues)
- Severity (mild, moderate, severe)
- Whether they took their capsules that day

Researchers call participants weekly to check in and ask about side effects.

7. Safety First

7.1 Inclusion Criteria (Who Can Participate?)

To join the study, a person must meet ALL of these:

- Age 50-75 years
- Generally healthy (no hospitalization in the past 6 months)
- BMI between 18.5 and 35
- Willing to take supplements as directed
- Willing to attend 5 study visits
- Willing to provide blood samples
- Able to give informed consent

7.2 Exclusion Criteria (Who Cannot Participate?)

A person CANNOT participate if any of these apply:

- Active cancer or cancer treatment within the past 5 years
- Pregnancy or breastfeeding
- Kidney disease (creatinine > 1.5 mg/dL)
- Liver disease (ALT or AST > 2× normal)
- Taking immunosuppressive drugs
- Taking blood thinners (warfarin, etc.) — fisetin may interact
- Allergy to any of the study compounds
- Currently taking fisetin, NMN, or high-dose spermidine supplements
- Participating in another clinical trial
- Substance abuse or uncontrolled psychiatric illness

7.3 Side Effects to Watch For

Compound	Potential Side Effects	Likelihood
Fisetin		

	Mild nausea, temporary diarrhea, rare allergic reaction	Low (no serious AEs in human trials at similar doses)
NMN	Mild flushing, slight nausea, insomnia if taken late in day	Very low (well-tolerated in human studies)
Spermidine	Mild digestive discomfort at high doses	Very low
Piperine	Mild stomach upset	Very low

7.4 Study Stopping Rules

The study will be immediately stopped if:

- Any participant experiences a Serious Adverse Event (SAE) that is definitely or probably related to the study supplements
- More than 3 participants experience the same moderate-to-severe adverse event
- The Independent Safety Monitor recommends stopping

7.5 Ethics

- The study protocol must be approved by an **Institutional Review Board (IRB)** or ethics committee BEFORE any participant is enrolled
- All participants must sign an **informed consent form** explaining the study, the compounds, and the risks in plain language
- Participants can leave the study at any time, for any reason, without penalty
- All data is anonymized (names replaced with codes)
- The study is registered on **ClinicalTrials.gov** before recruitment begins

8. Complete Equipment List

Here is everything you need to run this study, from lab equipment to supplements. Prices are estimates in USD and may vary by region, supplier, and brand.

8.1 Laboratory Equipment

Item	Purpose	Est. Cost (USD)
Microplate Reader (absorbance + fluorescence)	Reading ELISA plates for IL-6, IL-8, SDF1, NAD+ assays	$8,000 - $15,000
Real-time PCR (qPCR) machine	Measuring p16/p21 mRNA levels	$25,000 - $45,000
Centrifuge (refrigerated, benchtop)	Separating blood plasma/serum	$3,000 - $6,000
-80°C Freezer	Long-term storage of blood samples	$8,000 - $12,000
-20°C Freezer (standard)	Short-term storage of reagents and samples	$1,000 - $2,500
4°C Refrigerator	Storing ELISA kits, buffers	$500 - $1,500
Micropipettes (set of 4: P10, P100, P1000, P5000)	Precise liquid measurement	$600 - $1,200
Vortex mixer	Mixing samples	$200 - $500
Precision balance (0.001g)	Weighing supplements for encapsulation	$300 - $800
Microcentrifuge (small, benchtop)	Quick spins for small samples	$500 - $1,500
Water bath or heat block	Incubating ELISA plates, enzyme reactions	$300 - $800
pH meter	Preparing buffers	$100 - $300
Encapsulation machine (manual, capsule filler)	Filling capsules with precise doses	$200 - $600

8.2 Consumables

Item	Purpose	Est. Cost (USD)
ELISA kits — Human IL-6	Measuring IL-6 in blood serum	$450/kit (96 wells, ~40 samples)
ELISA kits — Human IL-8	Measuring IL-8 in blood serum	$450/kit
ELISA kits — Human SDF1/CXCL12	Measuring SDF1 in blood serum	$500/kit
NAD+/NADH assay kit	Measuring NAD+ levels in blood	$400/kit
p62 ELISA kit	Measuring autophagy marker	$450/kit
qPCR reagents (RT-qPCR kit)	For p16 and p21 mRNA measurement	$500 - $1,000/kit
qPCR primers (p16, p21, GAPDH)	Specific DNA sequences to detect target mRNA	$150/primer set
96-well microtiter plates	ELISA assays	$50/pack of 50
Pipette tips (filtered, assorted sizes)	Preventing contamination	$100/pack
Eppendorf tubes (1.5mL, 0.5mL)	Sample storage	$50/pack
Blood collection tubes (EDTA, serum separator)	Drawing and storing blood	$100/box of 100
Cryovials (2mL, for -80°C storage)	Long-term sample storage	$50/pack of 100
Disposable gloves (nitrile)	Safety	$30/box
Empty capsules (vegetarian, size 0)	Encapsulating supplements and placebo	$20/1000 capsules
Microcrystalline cellulose	Placebo filler	$20/kg

8.3 Supplements (GMP-Certified, for 40 participants × 16 weeks)

Compound	Quantity Needed	Est. Cost (USD)
Fisetin (GMP-grade, ≥98% purity)	20 participants × 14 pulse days × 500mg = 140g	$800 - $1,500

NMN (GMP-grade, ≥99% purity)	20 participants × 112 days × 250mg = 560g	$1,000 - $2,500
Spermidine (GMP-grade, ≥99% purity)	20 participants × 112 days × 3mg = 6.7g	$300 - $600
Piperine (standardized extract, 95%)	20 participants × 14 pulse days × 10mg = 2.8g	$50 - $100

Note: Only the treatment group receives real supplements. The placebo group needs the same amount of cellulose filler. Total supplement cost is roughly halved for the placebo group.

8.4 Clinical Assessment Equipment

Item	Purpose	Est. Cost (USD)
Digital camera + ring light (standardized photography)	Facial photography for brown spot analysis	$500 - $1,200
Hand dynamometer	Grip strength measurement	$200 - $500
Stopwatch (digital)	Walking tests, chair stands	$20
Measuring tape (4 meters, marked)	Gait speed test	$10
MoCA test sheets (printed)	Cognitive assessment	Free (downloadable) or $1/copy
SF-36 questionnaires (printed)	Quality of life assessment	Free (downloadable) or $1/copy
Blood pressure monitor	Basic vital signs	$50 - $100
Weighing scale (digital)	Body weight / BMI	$40 - $80
Stadiometer (height measurement)	Height for BMI	$100 - $200

8.5 Software

Software	Purpose	Cost
R (statistical computing)	t-tests, ANOVA, data visualization	Free (open source)
GraphPad Prism (optional)	Easier statistical analysis and graphing	$250 - $500/year

REDCap or Excel	Secure data storage (REDCap is free for academia)	Free
ImageJ (with skin analysis plugins)	Measuring brown spot area from photos	Free (NIH)

8.6 Total Estimated Budget

Category	Est. Cost Range (USD)
Lab equipment (one-time)	$47,000 - $86,000
Consumables (per study)	$3,000 - $6,000
Supplements	$2,000 - $4,700
Clinical equipment	$1,000 - $2,400
Software	$0 - $500
Personnel, IRB fees, participant compensation	$10,000 - $25,000
TOTAL (first study)	**$63,000 - $125,000**
Follow-up studies (equipment already purchased)	$15,000 - $35,000

9. Step-by-Step Timeline

Phase 1: Preparation (Weeks -8 to 0)

Week	Activity
-8 to -6	IRB/ethics submission and approval
-6 to -4	Order equipment, supplements, and supplies
-5 to -3	Recruitment: advertising, screening calls
-4 to -2	In-person screening visits (consent, blood test, medical history)
-2 to -1	Randomization (computer-generated assignment)
-1 to 0	Capsule preparation (encapsulation, labeling, blinding)
Day 0	Baseline visit (Visit 1): blood draw + clinical measurements

Phase 2: Treatment (Weeks 1-16)

Week	Activity
1	Treatment begins. Pulse Day 1-2. Distribute capsules and diaries.
2-3	Maintenance phase. Weekly phone check-ins.
4 (Day 30)	Visit 2: blood draw. Pulse Day 3-4.
6-7	Maintenance phase.
8 (Day 60)	Visit 3: blood draw. Pulse Day 5-6. Midpoint assessment.
10-11	Maintenance phase.
12 (Day 90)	Visit 4: blood draw. Pulse Day 7-8.
14-15	Maintenance phase.
16 (Day 120)	Visit 5: Final blood draw + full clinical assessment. Last pulse.

Phase 3: Analysis (Weeks 17-20)

Week	Activity
17-18	Lab analysis: run all ELISAs, qPCR, NAD+ assays on stored samples
18-19	Unblinding: reveal which participants were in which group
19-20	Statistical analysis, report writing
20	Results shared with participants. Manuscript preparation.

Simplified Gantt Chart

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Prep: [========] (Weeks -8 to 0) Treatment: [========================] (Weeks
1-16) Analysis: [====] (Weeks 17-20)
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10. Statistical Analysis Plan

10.1 The Basic Idea (in Plain English)

We have two groups of 20 people. One got the real treatment, one got the placebo. After 16 weeks, we compare their biomarkers. If the treatment group has significantly lower p16, lower IL-6, higher NAD+, etc., then the treatment works.

> **Simple Explanation**
>
> "Significantly" is the key word. Maybe the treatment group's p16 went down by 5%, but the placebo group's p16 also went down by 3% (random variation). Is the 2% difference real or just noise? Statistics tells us. We use the magic threshold of **$p < 0.05$**, which means: "there's less than a 5% chance this difference is due to luck alone."

10.2 Tests We Use

Between-group comparison at each time point

Independent samples t-test: compares the average p16 level (for example) between the treatment group and the placebo group at Day 30, Day 60, etc.

> **Simple Explanation**
>
> **t-test explained**: Imagine the treatment group has an average p16 of 10 and the placebo group has 15. The t-test asks: "Given the natural variation in both groups, how likely is it that these two averages could come from the same population?" If that likelihood is below 5% ($p < 0.05$), we conclude the difference is real.

Changes over time

Repeated-measures ANOVA (Analysis of Variance): compares how biomarkers change across all 5 time points (Day 0, 30, 60, 90, 120) between the two groups. This is more powerful than doing 5 separate t-tests because it looks at the overall pattern.

> **Simple Explanation**

> **ANOVA explained**: Instead of asking "is Day 120 different?", ANOVA asks "is the overall *trajectory* different?" If the treatment group's p16 smoothly decreases over 4 months while the placebo group stays flat, ANOVA captures that.

Effect Size

Beyond statistical significance, we report **Cohen's d** — a measure of how large the effect is:

- d = 0.2 → small effect
- d = 0.5 → medium effect
- d = 0.8 → large effect

A treatment can be statistically significant but practically meaningless (tiny effect on a huge sample). We want to see both significance AND a meaningful effect size.

10.3 Why 40 People Is Enough

This is a **pilot study**. Its goal is not to prove the treatment works definitively, but to see if there are *signals* worth pursuing in a larger trial. For pilot studies:

- 40 participants (20 per group) is the generally accepted minimum
- We can detect large effects (Cohen's d ≥ 0.8) with 80% power
- Smaller effects would need a larger follow-up study (100+ participants)
- If we see NO signal at all in 40 people, it's unlikely the treatment works
- If we see a strong signal, we write a grant for the definitive 200-person trial

10.4 Handling Missing Data

- Participants who drop out are analyzed in the group they were assigned to (intention-to-treat)
- Missing biomarker values are imputed using the last observation carried forward (LOCF)
- Adherence is tracked via capsule counting at each visit

11. Glossary

Apoptosis: Programmed cell death. The normal, clean way a damaged cell dies. Senescent cells resist apoptosis.

Autophagy: The cell's recycling system. Damaged proteins and organelles are broken down and reused. Declines with age.

Biomarker: A measurable substance in the body that indicates a biological state or condition (like p16 for senescence).

CXCL12: Another name for SDF1. A chemokine that regulates melanocyte behavior.

DDR (DNA Damage Response): The cellular alarm system that activates when DNA is damaged, triggering repair or senescence.

Double-blind: A study design where neither participants nor researchers know who receives the real treatment.

ELISA: A lab technique that uses antibodies and color change to detect and quantify specific proteins in blood.

Fisetin: A natural flavonoid found in strawberries, apples, and onions. The most potent natural senolytic known.

GMP (Good Manufacturing Practice): Quality standards for producing supplements and drugs. GMP-grade means the product is pure, consistent, and safe.

Hayflick Limit: The maximum number of times a human cell can divide (~50) before entering senescence. Discovered by Leonard Hayflick in 1961.

HPLC: A lab technique that separates and identifies molecules in a mixture. Used to measure NAD+ levels.

IL-6 / IL-8: Interleukins — inflammatory signaling molecules. Key components of the SASP.

Inflammaging: Chronic, low-grade inflammation that increases with age. Driven partly by the SASP from senescent cells.

IRB (Institutional Review Board): An ethics committee that reviews and approves research studies to protect participants.

LC3-II/I ratio: A marker of autophagy. High ratio = active recycling.

NAD+ (Nicotinamide Adenine Dinucleotide): An essential coenzyme for energy metabolism and DNA repair. Declines with age.

NMN (Nicotinamide Mononucleotide): A direct precursor of NAD+. Supplementing boosts NAD+ levels.

$p16^{INK4A}$: A protein that permanently stops cell division. The gold-standard biomarker of senescence.

p21: Another cell cycle inhibitor. Often activated before p16 (temporary arrest).

p53: The "guardian of the genome." Activates DNA repair or triggers senescence/apoptosis when DNA is damaged.

p62 (SQSTM1): A protein degraded during autophagy. Low levels indicate active autophagy.

Placebo: An inactive substance that looks identical to the real treatment. Used to distinguish real effects from psychological effects.

qPCR (quantitative PCR): A technique to measure the amount of specific mRNA (genetic messages) in a sample.

Randomization: Assigning participants to groups by chance (like flipping a coin) to eliminate bias.

ROS (Reactive Oxygen Species): Damaging molecules produced by cellular metabolism. Like "exhaust fumes" of the cell.

SASP (Senescence-Associated Secretory Phenotype): The toxic cocktail of inflammatory molecules secreted by senescent cells.

SCAP (Senescent Cell Anti-apoptotic Pathway): The survival network that prevents senescent cells from dying. Targeted by fisetin.

SDF1 (Stromal Cell-Derived Factor 1): A protein that regulates melanocytes. Low in senescent skin tissue (Yoon 2018).

Senolytic: A compound that selectively kills senescent cells.

Spermidine: A natural polyamine that induces autophagy. Found in wheat germ, shiitake mushrooms, aged cheese.

Telomere: Protective caps at the ends of chromosomes. Shorten with each cell division until the cell becomes senescent.

Tyrosinase: The enzyme that produces melanin (pigment). Abnormally active in senescent fibroblasts within brown spots.

Western Blot: A technique to detect and quantify specific proteins using antibodies and size separation.

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This protocol is a theoretical document for educational and research planning purposes. It is not medical advice. Any actual clinical study must be reviewed and approved by a qualified Institutional Review Board (IRB) and conducted under appropriate regulatory oversight. All supplements must be GMP-certified and tested for purity.

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Prepared with care for curious minds.

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